



## **Stable isotope probing and dynamic loading experiments provide insight into the ecophysiology of novel ammonia oxidizers in rapid gravity sand filters**

**Fowler, Jane; Palomo, Alejandro; Gülay, Arda; Tatari, Karolina; Thamdrup, Bo; Albrechtsen, Hans-Jørgen; Sørensen, Søren; Smets, Barth F.**

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### **An optimised toolbox for investigating free-living diazotrophs in soil: from bulk measurements to single-cell analysis**

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Biological nitrogen fixation is carried out by various bacteria and archaea (diazotrophs), which can be free-living or plant symbionts. Despite potentially low per-cell N<sub>2</sub> fixation rate, free-living diazotrophs are of great global importance since they are widely distributed in the environment. However, knowledge about the identity and ecophysiology of free-living diazotrophs is still limited. We developed a toolbox for studying active, free-living diazotrophs using <sup>15</sup>N<sub>2</sub> stable-isotope labelling, molecular tools and single-cell analysis techniques. Soil samples were incubated with <sup>15</sup>N<sub>2</sub>. The incorporation of <sup>15</sup>N was used to identify active diazotrophs and as a measure of diazotrophic activity. By tracing the accumulation of <sup>15</sup>N into different biomolecules, particularly RNA, detection of diazotroph activity occurred sooner than when bulk soil was analysed and as quickly as within one day of incubation. To identify active diazotrophs we developed an RNA-stable isotope probing assay (<sup>15</sup>N-RNA-SIP), which allows detecting <sup>15</sup>N-enriched diazotrophs within a few days of activity. Compared to DNA-SIP, this method could allow a more rapid identification of diazotrophs. By comparing the sequence abundance in different fractions of the density gradient, specific OTUs were identified as labelled using a differential expression model. Using this method, several OTUs classified as Clostridia, Bacilli, Proteobacteria and Actinobacteria were identified as active diazotrophs. The activity of diazotrophs identified by <sup>15</sup>N-RNA-SIP can then be verified using the FISH-NanoSIMS approach. In summary, these tools allow identifying and investigating active free-living diazotrophs in a highly sensitive manner in diverse environments, from bulk to the single-cell level.

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### **Stable isotope probing and dynamic loading experiments provide insight into the ecophysiology of novel ammonia oxidizers in rapid gravity sand filters**

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Nitrification is often the dominant microbial process in rapid gravity sand filters (RSF), used to treat aerated groundwater to produce drinking water. RSFs harbor diverse microbial communities including a range of ammonia oxidizing clades; Betaproteobacteria (*Nitrosomonas*, *Nitrosospira*), Archaea, diverse potentially ammonia oxidizing heterotrophs and abundant *Nitrospira* spp., recently shown to comprise both canonical nitrite oxidizing as well as complete ammonium oxidizing (comammox) types. We examined the contributions of the different ammonia oxidizers to *in situ* ammonia oxidation, and aimed to elucidate the differences in ecophysiology between the

ammonia oxidizing clades that enable them to co-exist in this unique environment. Experiments were conducted using sand columns designed and operated to mimic the conditions in the full-scale parent RSF. RNA and DNA stable isotope probing based on  $^{13}\text{C}$ -bicarbonate incorporation during continuous feeding with either ammonium or nitrite as sole energy source implicated *Nitrospira* spp. and certain 'heterotrophic' bacteria in addition to *Nitrosomonas* spp. in autotrophy during ammonium oxidation in RSFs. Further experimentation aimed to elucidate the ecophysiology of each ammonia oxidizing clade in RSFs, in particular comammox *Nitrospira* for which little is currently known. Columns were fed with RSF effluent spiked with various concentrations of ammonium ranging from 0.1- 5.0 mg/L delivered at different loading rates to examine the effects of both ammonium loading and oxygen limitation on ammonia oxidizers. Our observations indicate that the native conditions in the RSF used in this study foster the enrichment of comammox *Nitrospira*, which provides a preliminary step in the description of their ecophysiology.

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### **Dynamic, mechanistic, molecular-level modeling of cyanobacteria: *Anabaena* and nitrogen interaction**

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Phytoplankton models can provide important insights for ecosystem research and management. The existing models were originally developed in the 1970s based on simplified concepts (e.g., Monod kinetics) and generally have not been updated to include modern biology, which limits their utility. Here, we present a dynamic, mechanistic, molecular-level (i.e., gene, transcript, protein, metabolite) model of *Anabaena* – N interaction. The model simulates individual filaments, each with individual cells, each with genes that are expressed to yield transcripts and proteins. Cells metabolize various forms of N, grow and divide, and differentiate heterocysts (specialized cells that fix  $\text{N}_2$ ) when fixed N is depleted. The model is informed by observations from 269 laboratory experiments from 55 papers published from 1942-2014. Within this database, we identified 331 emerging patterns, and, excluding inconsistencies in observations, the model reproduces 94% of these patterns. Application to isotope tracing experiments suggests a role for an N storage pool, consistent with recent observations. To explore a practical application, we used the model to simulate nutrient reduction scenarios for a hypothetical eutrophic lake. For a 50% N-only loading reduction, the model predicts that  $\text{N}_2$  fixation increases, but the amount of N fixed does not compensate for the load reduction, and chlorophyll *a* concentration decreases substantially (by 33%). When N is reduced along with P, the model predicts an additional 8% reduction (compared to P only). These results are consistent with recent findings that, in most instances, dual (N+P) reductions are needed to control eutrophication on both short and long timescales.

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## Diazotrophs in West Philippine Sea under influence of mesoscale eddies

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Biological nitrogen fixation is an important source of new production in subtropical North Pacific Ocean. However, the community composition and limiting factors of the diazotrophs in the subtropical Western North Pacific Ocean (WNPO) is still unclear. In this study, we reported the diazotrophic community structure, as well as the abundance and activities of the important phylotypes in the highly dynamic West Philippine Sea (WPS) of the WNPO, using 454-pyrosequencing and quantitative PCR (qPCR). At community level, unicellular cyanobacteria UCYN-A2 was predominant in coastal water, while UCYN-A1 and *Gammaproteobacteria* γ-24774A11 were predominant in oceanic water. *Trichodesmium* was predominant in Kuroshio Current (KC), warm eddy and cold eddy. At cDNA level, *Trichodesmium* and γ-24774A11 contributed majority (> 90 %) of the *nifH* gene transcripts in the WPS. In addition, we have detected a bloom of *Trichodesmium* ( $10^8$  gene copies per liter) in the cold eddy, and the abundance of *Trichodesmium* was strongly and positively correlated with iron concentration ( $r = 0.84$ ,  $p$ -value < 0.05,  $n = 8$ ). Our results suggested that cold eddy may play an important role in the dynamic of nitrogen fixation process in the subtropical WNPO. Moreover, we suggest that diazotrophs in the WPS should be limited by iron availability.

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## Microbial functional genes related to soil nitrogen cycling in Swiss integrated organic cropping systems

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The series of soil microbial-mediated steps resulting in plant-available nitrogen and nitrogen gaseous losses via mineralization, nitrification and denitrification is of agronomic, economic and environmental significance. Nitrogen loss and application reduction in agriculture requires an understanding of how management practices drive the soil-microbe system. This study aimed to evaluate the effect of organic versus inorganic fertilization and conventional versus reduced tillage practices on nitrogen pool changes and denitrification potential and its link to microbial response via the abundance of genes related to these processes. To do this, mineralization, nitrification and potential denitrification incubations were carried out and abundance of *amoA* AOA, *amoA* AOB, *nirK*, *nirS* and *nosZ* genes were quantified. Although no significant differences were found regarding mineralization and nitrification, DNA content at incubation start tended to match with nitrate produced. Tillage had a significantly higher effect than fertilization on total bacterial content and *nirK* abundance during denitrification, where conventional plow was greater than no-till. On the other hand, *nirS* gene abundance was greater in the organic plow treatment highlighting a shift in bacterial nitrate reductase community composition. Although no significant effect was detected for potential and net N<sub>2</sub>O

emissions, the inorganic fertilizer in no-till treatment tended towards higher denitrification potential which can imply a higher environmental impact. The different fertilization and tillage treatments caused variation in microbial response which was statistically uncoupled from measured nitrogen pools, highlighting the need to further optimize approaches towards elucidating the effect of different management practices on nitrogen plant supply and system losses.

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### **Metabolic versatility of a cosmopolitan heterotrophic diazotroph isolated from a marine oxygen minimum zone**

Wiebke Mohr\*, Clara Martínez-Pérez, Anne Schwedt, Marcel M.M. Kuypers  
*Max-Planck-Institute for Marine Microbiology, Germany*

Recent findings suggest that marine oxygen-minimum zones (OMZs) are not only major areas of N loss but also hot-spots of N<sub>2</sub> fixation. *nifH* gene sequencing has revealed a large diversity of N<sub>2</sub>-fixers inhabiting the OMZs, however, little is known about the physiology of these organisms due to the lack of cultured representatives. Here, we present, to the best of our knowledge, the first diazotroph isolated from the OMZ off Peru in the South Pacific Ocean. Genome sequencing of the isolate indicates that it is a member of the Rhodobacteraceae family (Rhodobacterales,  $\alpha$ -Proteobacteria). The closed genome has a total size of 4.6 Mb and a GC content of about 65%. The isolate carries a complete set of *nif* genes as well as the *Rhodobacter*-characteristic *rnf* genes for N<sub>2</sub> fixation. Sequence analyses show the absence of any inorganic carbon fixation pathways indicating a heterotrophic metabolism. Further, both aerobic as well as anaerobic respiratory pathways are present, possibly to retain metabolic versatility during changing oxygen concentrations in the OMZ waters. Closely related *nifH* sequences have been recovered from a variety of marine environments indicating that this organism might be widespread in the ocean.

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### **Heterotrophic dinitrogen (N<sub>2</sub>) fixation persists throughout the euphotic zone of the South Pacific Gyre**

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*Max-Planck-Institute for Marine Microbiology, Germany*

Dinitrogen (N<sub>2</sub>) fixation is the largest source of bioavailable nitrogen (N) in the ocean, and as such exerts primary control on marine productivity. While some areas like the North Atlantic have been studied well with respect to N<sub>2</sub> fixation rates, the subtropical South Pacific has rarely been studied despite the fact that it is the largest gyre in the world's oceans. Here, we measured N<sub>2</sub> fixation rates along a transect through the subtropical South Pacific Gyre (SPG) from Chile to New Zealand during austral summer 2015/2016. N<sub>2</sub> fixation was measurable with low but significant rates all along the SPG except in waters influenced by the oxygen-minimum zone in the East. A vertical gradient was evident with measurable N<sub>2</sub> fixation rates in the euphotic zone and low to non-

detectable rates below the deep chlorophyll *a* maximum. Surprisingly, the colonial *Trichodesmium* sp. and the unicellular cyanobacterial group A (UCYN-A), which are the two globally most abundant N<sub>2</sub>-fixing microorganisms, were absent. Diatom-diazotroph associations were sporadically present in plankton net tows but only at very low abundances. In combination with previous studies, these results indicate that heterotrophic N<sub>2</sub> fixation might prevail in the SPG. To our knowledge, this is the first comprehensive, quantitative dataset on N<sub>2</sub> fixation rates in the subtropical South Pacific Gyre, the largest oligotrophic biome of the oceans with potentially enormous impacts on global biogeochemistry.

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### **Characterizing new vs regenerated production and the organisms involved through stable isotope probing at the San Pedro Ocean Time-Series**

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New versus regenerated production (NvR), production driven by allochthonous versus recycled nitrogen, has long been used to characterize marine environments. Understanding the role individual organisms play in these processes is crucial to understanding the dynamics of nitrogen cycling and ultimately carbon sequestration. However directly linking these processes to specific organisms can be difficult. Our project attempts to help bridge the gap between microbial ecology and biogeochemistry by employing multiple tracer methods to compare the rates of several key processes involved in NvR, i.e. <sup>15</sup>N-NO<sub>3</sub><sup>-</sup>, <sup>15</sup>N-NH<sub>4</sub><sup>+</sup>, and <sup>15</sup>N-urea uptake, as well as identify organisms participating in the transformation and assimilation of these substrates. The coupling of stable isotope probing (SIP) with high throughput tag-sequencing (TAG-SIP) was used to examine the incorporation of labeled substrate into individual taxa during April at the San Pedro Ocean Time-series (SPOT), facilitating an in-depth investigation of the activity and functional diversity of the *in situ* prokaryotic and eukaryotic communities at several light levels. Regenerated production, primarily through urea assimilation, appears to be a major source of nitrogen driving activity during this month at this site, largely in the two higher light levels. The eukaryotic community, particularly diatoms, were identified as the primary utilizers of this substrate through SIP, though many heterotrophic prokaryotes were also shown to utilize urea. Several OTUs displayed evidence of assimilation of multiple substrates at multiple depths while others appear to be more specialized on a single substrate. This unique insight into functional microbial ecology can be used to better understand ecosystem dynamics.

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### **Diversity and phylogenetic distribution of extracellular microbial peptidases**

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Depolymerization of proteinaceous compounds by extracellular proteolytic enzymes is a bottleneck in the nitrogen cycle, limiting the rate of the nitrogen turnover in soils. Protein

degradation is accomplished by a diverse range of extracellular (secreted) peptidases. Our objective was to better understand the evolution of these enzymes and how their functional diversity corresponds to known phylogenetic diversity. Peptidase subfamilies from 110 archaeal, 1,860 bacterial, and 97 fungal genomes were extracted from the MEROPS database along with corresponding SSU sequences for each genome from the SILVA database, resulting in 43,177 secreted peptidases belonging to 34 microbial phyla and 149 peptidase subfamilies. We compared the distribution of each peptidase subfamily across all taxa to the phylogenetic relationships of these organisms based on their SSU gene sequences. The occurrence and abundance of genes coding for secreted peptidases varied across microbial taxa, distinguishing the peptidase complement of the three microbial kingdoms. The distribution of secreted peptidases was found to be significantly correlated with phylogenetic relationships within kingdoms (archaea  $r_{\text{Mantel}}=0.364$ ,  $p=0.001$ ; bacteria  $r_{\text{Mantel}}=0.257$ ,  $p=0.001$ , and fungi  $r_{\text{Mantel}}=0.281$ ,  $p=0.005$ ), inferring an evolutionary relationship where subsets of phylogenetically related organisms share similar types of secreted peptidases. About one-third of the peptidase subfamilies displayed a strong evolutionary signal; the rest were phylogenetically over-dispersed, suggesting that these subfamilies are randomly distributed across the tree of life or the result of events such as horizontal gene transfer. Study of the diversity and phylogenetic distribution of secreted peptidases offered a mechanistic basis to anticipate the proteolytic potential function of microbial communities.

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### **How the ability to release exoenzymes may affect rates of nitrogen fixation by free-living soil diazotrophs**

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Through biological N fixation, prokaryotic microorganisms known as diazotrophs can access the functionally-infinite pool of atmospheric N gas to satisfy cellular N demand. Free-living diazotrophs, which can fix N outside of plant associations, are responsible for a large proportion of observed biological N fixation in terrestrial systems. Due to the high metabolic costs associated with N fixation, free-living diazotrophs only fix N under certain conditions in pure culture and these organisms likely rely on soil N to fill a high proportion of cellular N demand. Culture-based studies and genomic evidence show that many free-living diazotrophs can access high-molecular-weight N by releasing proteases and chitinases into the extracellular environment. We seek to understand how the ability to release N-acquiring exoenzymes affects rates of N fixation by terrestrial free-living diazotrophs by investigating the N acquisition strategies these organisms employ in the presence of high-molecular-weight N. Based on both cost-benefit principles and evidence from the literature, we propose that free-living diazotrophs in soils access available N pools in the following order: 1) low-molecular-weight N, 2) Atmospheric N, 3) high-molecular-weight N; we refer to this nitrogen acquisition strategy as the "LAH N-acquisition strategy" based on this order. Here we formally introduce the LAH N-acquisition strategy, discuss pure-culture evidence supporting this behavior from scientific literature, present novel experimental work testing the N acquisition strategies of free-living diazotrophs in pure culture, and make predictions about the potential

ecosystem-level effects of diazotrophic adherence to the LAH N-acquisition strategy in terrestrial systems.

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### **Imaging carbon, nitrogen and phosphorus assimilation by colonial cyanobacteria during a harmful algal bloom**

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Harmful algal blooms (HABs) of colonial N<sub>2</sub>-fixing cyanobacteria belonging to the genera *Nodularia*, *Dolichospermum* and *Aphanizomenon* are a recurrent phenomenon in the Baltic Sea during summertime. It has been suggested that these HABs are triggered by excess phosphate (P), and collapse at the end of summer as a result of P limitation. We used imaging techniques to investigate how carbon, nitrogen and phosphorus assimilation by the three N<sub>2</sub>-fixers responded to P-limitation in the Baltic Sea in the summer of 2015. *In situ* incubations with hand-picked colonies and radiolabeled phosphate revealed that a drop in phosphate concentrations below ~150 nmol/L leads to a several fold decrease in phosphate uptake rates by the N<sub>2</sub>-fixers. In contrast, single cell activity measurements using nanoSIMS showed that CO<sub>2</sub> and N<sub>2</sub> fixation rates were unaffected even when phosphate concentrations dropped to ~30 nmol/L. Dissolved organic phosphorus concentrations remained stable at ~500 nmol/L throughout the experiment indicating that organic P did not support the observed growth. In all three genera of colonial N<sub>2</sub>-fixers, the presence of P-storage granules was revealed by nanoSIMS and SEM-EDX measurements. We suggest that P-storage support growth of the N<sub>2</sub>-fixers under P-limiting conditions and plays a key role in determining the success of N<sub>2</sub>-fixing cyanobacteria during summertime HAB events in the Baltic Sea.

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### **Effects of nitrogen contaminated shallow groundwater on nitrogen metabolic microorganisms and its biochemical remediation**

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Groundwater contamination is a growing concern in China. Wastewater and solids produced by livestock and poultry breeding are discharged to the environment without any treatment, and seepage from the anaerobic swine lagoons is often percolated into groundwater. Meanwhile, treatment of polluted groundwater is difficult and costly. Thus how to effectively remove nitrogen from groundwater is still requires more research. This study investigated the effects of groundwater nitrogen contamination on nitrogen metabolism microorganisms, then analyzed the correlations between them, and designed a Multi-Soil-Layering to remove the nitrogen in groundwater by biochemical



remediation in groundwater. The main results are as follow: (1) The main nitrogen form in groundwater was  $\text{NH}_4^+\text{-N}$  in study site and showed spatial-temporal heterogeneity; (2) The main nitrogen metabolism bacteria in this site were ammonia oxidizing bacteria, nitrite reducing bacteria and nitrous oxide reducing bacteria. The dominant species was  *$\beta$ -Proteobacteria*; (3) The soil layers of the traditional MSL were replaced by organic layers that were a mix of saw dust and bran embedded with denitrification incula. Results showed that in the initial running period, almost all the nitrogen in groundwater was removed by strong sorption of the system. But after reached the sorption balance, nitrogen metabolic microbial contributed mainly to nitrogen removal in the polluted groundwater. The main types of nitrogen metabolic microbial were ammonia oxidizing bacteria, nitrous oxide reductase bacteria and ammonia oxidizing archaea. The dominant microbe in the system was  *$\beta$ -Proteobacteria*.

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### **Abundance and diversity of nitrogen-fixing bacteria in termite and cockroach guts**

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Nitrogen fixation by the gut microbiota is an important nitrogen source for wood-feeding termites, but the identity of the bacteria responsible for the activity and their numerical abundance among the gut microbiota is largely obscure. In this study, we used high-throughput sequencing of amplified *nifH* genes to characterize the potential diazotrophs in various termite families and in related cockroaches, which considerably increased taxon sampling and diversity coverage over previous studies. Ordination analyses showed a clear clustering of the diazotrophic communities among cockroaches (*Blattidae*) and higher termites (*Termitidae*). A discrete clustering was also obtained for lower termites, although they were not fully resolved at family level. While the majority of the *nifH* sequence reads fell into the radiation of classical nitrogenases (mostly Group III), a relatively large proportion belonged to Group IV, whose function in nitrogen fixation is just emerging. In lower termites, many *nifH* phylotypes clustered with homologs of bacteria associated with termite gut flagellates, corroborating previous evidence for their importance in nitrogen fixation in certain lower termites. In higher termites, the most abundant phylotypes were assigned to homologs in fiber-associated bacteria (*Fibrobacteres* and TG3 phylum). The low ratio of *nifH* genes to 16S rRNA genes in most termites, determined by qPCR, suggests that only a surprisingly small number of gut microbes are diazotrophs. Further studies will have to address the relative expression levels of different *nifH* homologs and the basis of the highly divergent nitrogen-fixing activities in different termite species.

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### **Analysis of transcriptional regulatory mechanisms for nitrogen fixation genes in *Acidithiobacillus ferrooxidans***

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*Acidithiobacillus ferrooxidans* is an acidophilic iron-oxidizing autotroph with the ability to fix atmospheric nitrogen, which allows this bacterium to survive in extremely nutrient-poor environments, e.g., acid-mine drainages. It is known that microbial nitrogen-fixing activities are tightly repressed by easily available nitrogen sources, such as ammonium and amino acids, including glutamine. Although the annotated genome of *A. ferrooxidans* suggests that this bacterium has putative regulatory genes for nitrogen fixation, these are distinct from those in well-characterized heterotrophic diazotrophs, and detailed molecular mechanisms remain unexamined. To investigate regulatory mechanisms for nitrogen fixation in *A. ferrooxidans*, we characterized the nitrogenase activity and transcriptional profiles of *A. ferrooxidans* ATCC 23270 in the presence or absence of ammonium. Measurements of the nitrogenase activity by acetylene-reducing assays revealed that ATCC 23270 cells express the activity only under ammonium-depleted conditions. Transcriptome analysis using DNA microarrays confirmed that the structural genes for nitrogenase, *nifHDK*, were markedly up-regulated under ammonium-depleted conditions. We also found that the nitrogenase activity and expression of the nitrogenase genes were enhanced, when *nifA*, the putative transcriptional activator gene flanking to *nifH*, was overexpressed. The *nifA*-overexpressing strain also exhibited an increased growth rate compared to the wild-type strain under ammonium-depleted conditions. These results indicate that the nitrogen-fixation activity of *A. ferrooxidans* is tightly regulated by the availability of nitrogen sources, and transcriptional activation of the nitrogenase genes by NifA is the key mechanism for controlling the nitrogenase activity of this bacterium.

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